Long range restriction analysis of the bovine casein genes

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Received September 27, 1990; Revised and Accepted November 9, 1990

ABSTRACT

Pulsed field gel electrophoresis (PFGE) was used to analyse the organization of the bovine αs1, αs2, β and κ casein genes. High molecular weight DNA was prepared from fibroblasts and lymphocytes embedded in agarose and was digested with the restriction endonucleases ClaI, SalI, SmaI, XhoI. The digestion products were separated by PFGE, transfered to nitrocellulose filters and hybridized to probes corresponding to the cDNAs of the four bovine casein genes. The casein genes were demonstrated to be physically linked within a region of 300 kb, represented by two adjacent XhoI fragments in fibroblasts and by a single fragment in lymphocytes. A restriction map of the casein locus was derived and the order of the genes was shown to be κ, αs2, β, αs1.

INTRODUCTION

Casein genes have been studied intensively because of their highly regulated expression (tissue-, stage-, sex- and age-specific), and because, in domestic animals, they have a major impact on milk and cheese production (1). In cattle four casein genes are known, αs1, αs2, β and κ. Polymorphisms have been demonstrated for all of them, except the αs2; the polymorphisms are useful for the identification of alleles associated with a higher protein yield or with specific dairy properties, like the κ-casein B in the bovine species (2−4).

All the cDNAs corresponding to the bovine casein genes are available (5) and recently the organization and sequence of the β and κ genes have been reported (6−8).

It is well established that the αs1, αs2 and β form a small gene family and, although the κ is unrelated to the others (7,8), a tight genetic linkage has been demonstrated for all the casein genes (9,10). In addition, the genes have been mapped recently to chromosome 6 (11). Nevertheless, attempts to find overlaps between cloned genomic sequences of the casein genes in cattle and rat have been so far unsuccessful.

PFGE is a recent technique that allows the separation of DNA fragments of up to 10−12 megabases (reviewed in 12,13). Following PFGE, Southern blotting and hybridization have been used to establish long range restriction maps of cluster of genes and of chromosomal regions in man, as well as of entire genomes in lower species (14−21). PFGE is also a powerful tool for the construction of physical maps of single chromosomes (22,23).

PFGE can be applied to demonstrate the linkage of genes and DNA markers directly at the DNA level, filling in the gap between techniques such as chromosome walking and *in situ* hybridization.

PFGE and Southern blotting were used in this study to demonstrate the physical linkage of the four bovine casein genes, to establish a long range restriction map of the region and to derive the order of the genes.

MATERIALS AND METHODS

Cells

Fibroblasts were from primary cultures of calf kidney. Frozen aliquots were thawed and grown in Ham's F12 medium supplemented with 20% fetal calf serum. Lymphocytes from fresh perypheral blood were purified on Ficoll gradients.

Preparation of high molecular weight DNA in agarose plugs

Cells, either trypsinized fibroblasts from cultured monolayers or lymphocytes from perypheral blood, were washed extensively with 1× PBS (Posphate buffered saline) and concentrated to 2−3 10^7/ml. Inclusion in agarose plugs (0.1 ml each) and cell lysis were according to published procedures (12).

Restriction enzyme digestions

Plugs were equilibrated O/N at 4°C and then washed 3 times (about 30′ per wash) in 10 vol. of 10 mM TrisCl, 1 mM EDTA, pH8 (TE). Pieces were cut from the plugs (5 μg of DNA each piece) and transfered into 1 ml of the appropriate restriction enzyme buffer for 30′ in ice. The buffer was removed and replaced with the enzyme mix containing 0.1 mg/ml acetylated BSA, 2 or 5 mM spermidine (respectively with 50 mM or ≥100 mM NaCl in the restriction buffer) and 20−40 units of endonuclease in a total volume of 0.1 ml. After 10′ in ice the tubes were moved to the recommended incubation temperature for 4 hours. In the case of double digestions, the same procedure was followed, except that the plugs were rinsed extensively with TE after the first enzyme treatment. Digestions were stopped by chilling in ice, removing the enzyme mix, washing with TE and

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placing the plugs into 1 ml of 20 mM Tris-Acetate, 11 mM EDTA, pH8.3 (STOP buffer) containing Orange-G.

PFGE

A Pharmacia Pulsaphor 2015 system was used with a CHEF hexagonal electrode (24). Gels, 1.5% agarose (SeaKem GTG, FMC), were cast and run in 0.5× TAE (20 mM Tris-Acetate 1 mM EDTA) at 190V, 240 mA, 16°C, for 18 hours. Plugs were sealed in the wells with 0.5% InCert agarose (FMC) in 0.5× TAE.

Molecular weight (MW) markers were lambda concatamers (FMC) and yeast *S.cerevisiae* strain X-2180-1B chromosomes prepared according to published procedures (12).

Blotting, hybridizations and probes

Gels were stained with ethidium bromide, destained in water and photographed with a UV light at 300 nm. Blotting was onto nitrocellulose (Hybond-C extra, Amersham; BAS85, Schleicher & Schuell) as described (25). Filters were hybridized in formamide (26). Washing was in 2× SSC, 0.1% SDS, 60°C, for 15 min. Filters were used for up to eight times after removal of the probes.

Purified fragments corresponding to the entire cDNAs of the casein genes, or to portions thereof, were used as probes (5). Labeling was performed with a random priming commercial kit (Multiprime, Amersham) and [α^{32}P]dCTP.

RESULTS

Physical linkage of the bovine casein genes

DNA from fibroblasts was digested with NotI, SfiI, NarI and SalI. Following PFGE and blotting, filters were hybridized to the κ, β, αs1 and αs2 probes. All revealed specific fragments $\geq$1,000 kb in the NotI, SfiI and NarI digests, whereas identical fragments of 560, 760, 850, 930, 1,000 and >1,000 kb were detected in the SalI samples (not shown). The 560 kb SalI fragment is shown in Fig.2; the products of higher MW migrated all together at the limiting mobility region, just above the 560 kb. Thus, the entire region of the casein genes was contained within the 560 kb SalI fragment.

A restriction map of the casein region

Additional enzymes were used to investigate the structure of the region. Table I shows the size of the fragments detected by the probes in the digests with SalI, ClaI, XhoI, and SmaI. Fig.1 is the map of the casein region based on the data hereafter described.

Some of the digestion products revealed in fibroblasts are shown in Fig.2. A 215 kb XhoI fragment was found with the αs2 and κ probes (the latter not shown) and a 120 kb one with the αs1 and β. All the probes identified a 335 kb XhoI fragment, probably resulting from incomplete cleavage at the XhoI site that joins the two XhoI end-products. A most likely explanation for the observed incomplete cleavage is that XhoI, like other 'rare cutters', was inhibited by C-methylation at the CpG dinucleotide of the target sequence, a modification particularly frequent in the genomes of higher eukaryotes.

With all the probes, double digestions performed with XhoI and SalI produced patterns identical to the single XhoI digestions, suggesting that the 215 and 120 kb fragments resided completely within the 560 kb SalI and contained the entire casein region (Fig.2).

ClaI, either singly or in combination with XhoI, helped locating

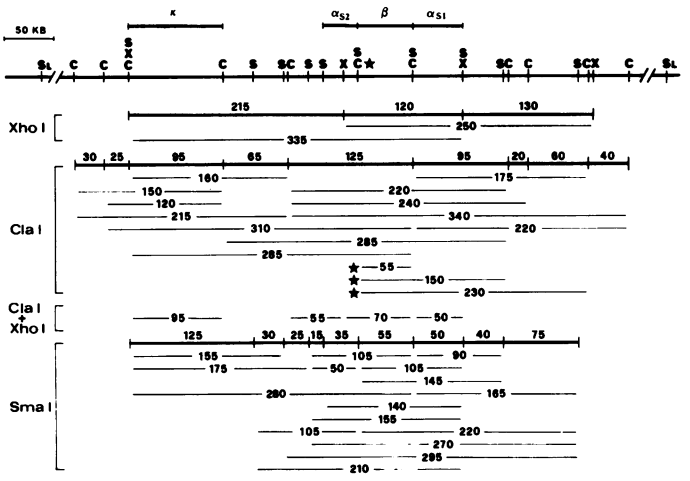

Fig.1 Physical map of the bovine casein locus. Restriction sites: C, ClaI; S_L, SalI; S, SmaI; X, XhoI. The star indicates the ClaI site that was cleaved only in lymphocytes and the resulting fragments. The XhoI sites indicated in the figure are derived from the analysis of fibroblsts; in lymphocytes the XhoI pattern presents a single 310 kb fragment (see Fig.3, RESULTS and DISCUSSION). Fragments detected by the probes are shown below the map with the indication of the MW.

the casein genes on the two adjacent XhoI fragments. In particular, the αs2 and β probes showed identical profiles, consisting of fragments of 125, 220, 285, 310 and 340 kb (Fig.2). Since the αs2 and β probes detected the 215 and 120 kb XhoI fragments respectively, the 125 kb ClaI band encompassed the XhoI site separating the 215 and 120 XhoI fragments. Consequentely, the fragments of higher MW in the profile originated from incomplete cleavage at sorrounding ClaI sites (Fig.1). In ClaI-XhoI digests the αs2 detected a 55 kb fragment and the β a 70 kb one, thus confirming that the two probes resided on the same 125 kb ClaI fragment (Fig.2). The αs1 was positioned to the right of the β since it revealed in the same ClaI-XhoI digest a 50 kb fragment, which represents the remaining portion of the 120 kb XhoI fragment that hybridized to the β and αs1 probes (Fig.2 and 1).

The interpretation of the patterns obtained in fibroblasts with the κ probe raised the possibility that the κ and αs1 casein genes might be on the same 95 kb ClaI fragment (Table I, Fig.2). This was shown not to be the case because 1) the αs1 and β, but not the αs2 or κ probes, detected a 250 kb XhoI fragment in addition to the already mentioned 120 and 335 kb (Fig.2) which suggests that there must be a 130 kb XhoI fragment to the right of the 120 kb one (Fig.1), 2) only the 95 kb ClaI fragment carrying the αs1 contains an XhoI site that produces the already mentioned 50 kb fragment in the ClaI-XhoI digests (Fig.2). Thus, the αs2 and κ resided on the same 215 kb XhoI fragment. In order to position the 95 kb ClaI fragment detected with the κ probe, the products of the partial ClaI digestions obtained were analyzed for fragments shared between the αs2 and κ probes.

SmaI digests of fibroblast DNA showed several fragments in the low MW range (from 35 to 155 kb) with the αs1, β and αs2 probes (Fig.2, Table I). Most of these fragments originated from partial cleavage, caused by C-methylation at the relevant sites, since the pattern was independent on the amount of enzyme used and the conditions of digestion. The comparison of the bands detected with the αs2, αs1 and β probes allowed the mapping of the Sma I sites that originated the partials of 105, 140, 145 and 155 kb (Fig.1 and 2).

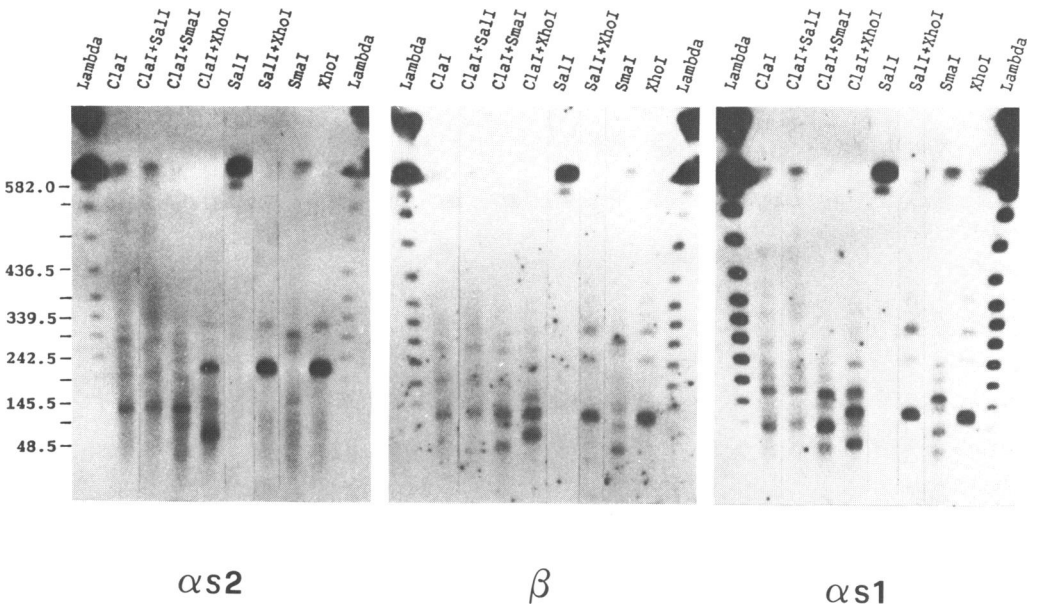

Fig.2. Long range restriction analysis of the bovine casein locus in fibroblats. Digests of DNA in plugs were separated in a CHEF exagonal array apparatus. The gel was run with pulses of 30 sec. and transfered to nitrocellulose. The same filter was hybridized to the cDNAs of the casein αs2 , β and αs1 as indicated, with the probe stripped between each hybridization. Numbers on the left indicate the size in kb of lambda concatamers used as MW standards.

Table I. Approximate Sizes of Restriction Fragments Detected by the Casein Probes

Probes	Restriction Enzymes					
	XhoI (L)	XhoI (F)	ClaI	SmaI	SalI	ClaI+XhoI
		120	95 150#	50 90	560	50
		250	175 215	105 140	760	120
αs1	310	335	230#240	155 165	850	
		360	285 340	220 240	930	
		410			1,000	
					≥1,000	
		215	125 220	35? 50	as αs1	55
αs2	310	335	210 270	105 140		215
			285 310	140 155		
			295			
			340			
		120	55#125	55 105	as αs1	70
		250	150#220	140 155		120
β	310	335	230#285	215 270		
		360	310 340	295		
		410				
		215	95 120	125 155	as αs1	95
κ	310	335	150 160	175		215
			215 285			
			310			

The fragments were detected by the four casein probes in fibroblasts and lymphocytes with the following exceptions: # indicates fragments visible in lymphocytes in addition to the others; 2) in the case of XhoI two columns are reported because in lymphocytes (L) a single fragment of 310 kb was observed with all the probes instead of the complex pattern of fragments visible in fibroblasts (F). See RESULTS and DISCUSSION for more details.

In the case of the κ probe no SmaI sites could be mapped in fibroblasts, although a very faint band of about 120−140 kb was visible in the SmaI digest (not shown). In addition the ClaI and the SmaI-ClaI digests showed identical patterns, suggesting that the smallest SmaI fragment hybridizing to the κ probe indeed might be larger than 95 kb.

In order to confirm the position of the sites derived from fibroblasts, DNA from lymphocytes was used as an alternative source. The results of the digestions performed with ClaI, SmaI, XhoI are shown in Fig.3. The patterns obtained confirmed the SmaI sites mapped in fibroblasts for the αs1, αs2 and β probes and helped locating additional SmaI sites mostly around the κ probe, that was shown to hybridize to fragments of 125, 155 and 175 kb. Again the pattern produced by the αs1 was different from

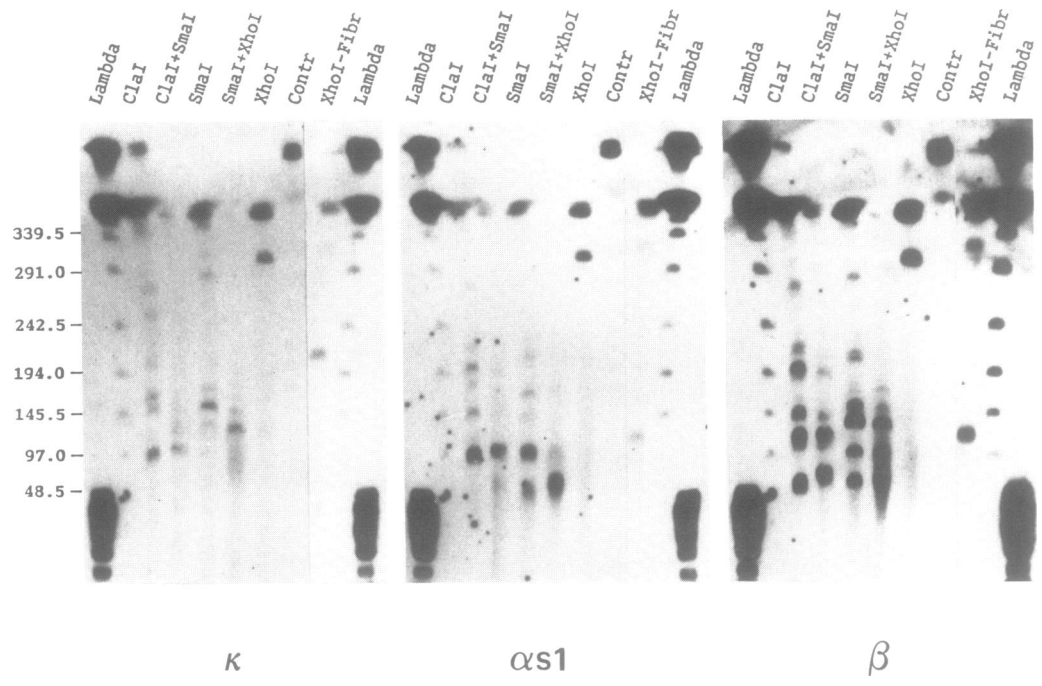

Fig.3. Long range restriction analysis of the bovine casein locus in lymphocytes. Conditions and MW standards are as in Fig.2, except that pulse time was 15 sec, and probes were κ, αs1 and β. XhoI-Fibr is a digest of fibroblast DNA; Contr is a control sample incubated in restriction buffer lacking enzyme.

the κ, confirming that the two genes did not map close to each other.

The ClaI pattern in lymphocytes was similar to those observed in fibroblasts, except for an additional site located within the 125 kb fragment detected by αs2 and β. This caused the appearence of a 55 kb ClaI fragment in the hybridization to the β probe (Fig.3) and was therefore mapped as shown in Fig.1.

The XhoI pattern in lymphocytes, shown in Fig.3, was interesting in that all the probes detected a single fragment of about 310 kb in place of the 120 and 215 kb, typical of the digestion of fibroblast DNA. A direct comparison of the 'lymphocyte' 310 kb with the 'fibroblast' 335 kb, resulting from partial cleavage at the XhoI site separating the 120 and 215 kb XhoI fragments, demonstrated that the products were indeed different.

DISCUSSION

In this study physical linkage of the four bovine casein genes was demonstrated by means of PFGE. The data presented are consistent with the recent localization of all the genes on chromosome 6 (13) and suggest a maximum size of 300 kb for the casein region. Noticeably a map of the casein genes could be derived by employing just two restriction enzymes, namely ClaI and XhoI. In addition the order of the genes in the cluster was shown to be κ, αs2, β, αs1.

In the map obtained the κ gene is at one end and is separated from the others by at least 70 kb, that could be 120−130 if the κ was close to the leftmost part of the 215 kb XhoI fragment shown in Fig.1. This feature of the map further supports the well established unrelatedness of the κ casein gene to the other three (8). On the contrary the αs2, β and αs1 genes are known to form an evolutionarily related family and the data presented show that the three genes span a region of about 130 kb.

Differences in the patterns generated by the enzymes ClaI, SmaI and XhoI in fibroblats and lymphocytes were observed. Any correlation with the expression of the casein genes was excluded *a priori* since in both tissues they are considered to be inactive. In the case of ClaI and SmaI, the diversities could be explained assuming a different methylation level at the mapped sites: the high sensitivity of the enzymes to methylation of the target sequence is well known. Infact, all the fragments detected with the probes both in fibroblasts and in lymphocytes could be positioned in the map of Fig.1 without inconsistencies. A 55 kb ClaI fragment was revealed by the β probe in lymphocytes that was totally undetectable in fibroblasts. It is unlikely that the event is caused by a rearrangment in the region, unless it is of moderate entity, since the overall structure of the map is the same in the two types of cells. We favour the hypothesis that the differences in the methylation level at that particular ClaI site are at a maximum between fibroblasts (complete methylation), and lymphocytes (substantial under-methylation).

A different case are the XhoI patterns where different digestion products are detected by the probes. Distinct methylation patterns might exist at the XhoI sites in lymphocytes and fibroblasts, so that sites mapped in one case could be totally undetectable in the other, as for the ClaI site just mentioned, thus giving rise to completely different products upon digestion with the enzyme. But a polymorphism at one of the XhoI sites flanking the casein region might explain the data as well, and indeed both the hypotheses could be true. It is possible that the appearance of a single XhoI fragment of altered MW could be the result of a rearrangment occurring in lymphocytes at the casein locus. We have no data to support this hypothesis, but we consider it unlikely because 1) the rearrangment should cause the disappearance of the XhoI site joining the 215 and 120 kb fragments visible in fibroblasts (Fig.1) 2) at least another external XhoI site should be removed 3) the digestion products obtained with the other

enzymes used in our analysis (ClaI and SmaI) either singly or in combination, are substantially the same in fibroblasts and lymphocytes.

The identification of a single XhoI fragment of about 310 kb hybridizing to all the casein probes in lymphocytes is an interesting finding that might be exploited to isolate and characterize the sequences involved in the coordinated expression of the casein genes. Work is in progress both to identify YACs harboring those sequences (27) and to clone the locus into YACs following preparative PFGE (28, 29) or into bacterial vectors (30,31).

ACKNOWLEDGMENTS

The cDNAs corresponding to the bovine casein genes were the gift of A.G.Mackinlay and S.I.Gorodetsky. The research was supported by CNR with grants in the P.F. 'Ingegneria Genetica' and 'Biotecnologie'.

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